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Immune Response Induced by Three *Mycobacterium bovis* BCG Substrains with Diverse Regions of Deletion in a C57BL/6 Mouse Model[▽]

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This study was performed to examine the adaptive immune response generated by three *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) substrains to determine if the number of genomic regions of deletion played a significant role in determining the magnitude of the immune response or affected their ability to reduce the bacterial burden following low-dose aerosol challenge with a virulent *M. tuberculosis* strain. BCG Connaught, Pasteur, and Sweden were chosen as representative substrains, as they possessed many, intermediate, and few regions of deletion, respectively, as a result of changes in the genome in various regions. Mice were vaccinated subcutaneously and were then examined at 14, 21, and 42 days postvaccination. BCG was observed in the spleen, lung, and lymph nodes. BCG Connaught induced a greater pulmonary T-cell response than the other two substrains at day 14 postvaccination, although by 42 days postvaccination activated T-cell levels dropped to the levels observed in control mice for all three substrains. Among the three substrains, BCG Connaught induced significantly greater levels of interleukin-12 in bone marrow-derived macrophage cultures. Mice challenged at days 14, 21, and 42 postvaccination displayed an equal capacity to reduce the bacterial burden in the lungs and spleen. The data provide evidence that although the BCG substrains generated qualitatively and quantitatively different immune responses, they induced similar reductions in the bacterial burden against challenge with a virulent *M. tuberculosis* strain in the mouse model of tuberculosis. The data raise questions about the assessment of vaccine immune responses and the relationship to a vaccine's ability to reduce the bacterial burden.

Mycobacterium bovis bacillus Calmette-Guérin (BCG), which was developed at the beginning of the 20th century, has been used worldwide as a vaccine against tuberculosis (24) and has efficacies that range from 0 to 80%, as reported from clinical trials (5, 12). The theories for this variability include interference and masking by environmental mycobacteria that are endemic in certain regions of the world. Genomic differences have been identified among the BCG substrains from different geographical sites that are used as vaccines due to propagation of the original parent strain at the various sites around the world (6, 7). It has been hypothesized that BCG may have been attenuated as a result of processing in the laboratory as part of vaccine preparation by different manufacturers and has been attenuated to such a degree that it has lost its potency at inducing a protective immune response (12). Analysis performed with a large number of BCG substrains has led to the identification of multiple genomic deletions, referred to as regions of deletion (RDs), in many substrains of BCG (1). As a result, BCG substrains can be typed on the basis of their RDs (2–4), although there is no clear evidence to support the theory that these RDs have any effect upon the efficacy of BCG as a vaccine. Despite the limited efficacy of the current BCG vaccine, there is continued interest because it is easy to produce, it is well tolerated, it possesses its own adjuvant, and there is evidence that a live vaccine may be needed to combat tuberculosis. Thus, it is necessary to identify the immune response generated by BCG and, possibly, why it fails to induce long-lasting immunity. BCG is currently being developed as a vehicle to carry immunogenic tuberculosis antigens and has been genetically modified to escape the phagosome of antigen-presenting cells in an attempt to make it a more efficacious vaccine (19, 30). In addition, the current effort to utilize novel candidates following BCG immunization as a prime/boost regimen is gaining momentum (21); therefore, it is necessary to identify how the differences that are seen at the genomic level affect the type or magnitude of the immune response and whether a particular strain of BCG would be preferred for use.

Given the different deletions present in different BCG substrains, we wanted to determine if the resulting loss of antigens negatively affected the immunogenicities of the strains or their ability to reduce the mycobacterial burden after a low-dose aerosol infection. In general, $CD4^+$ and $CD8^+$ T cells appear to be required for the effective control of mycobacterial growth, with the induction of key cytokines such as gamma interferon (IFN-γ) (10, 13). The mouse model of tuberculosis has been used extensively to define key factors of the immune response to mycobacterial infection, many of which have been confirmed in studies of the human disease (23). BCG vaccination in the mouse model has become the "gold standard"

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TABLE 1. *M. bovis* BCG substrains used in the current study[a]

M. bovis BCG substrain	RD(s)	Presence of Mpt64
Pasteur	RD1, RD2, nRD18, RD14	−
Sweden	RD1	−
Connaught	RD1, RD2, nRD18, RD8	+

[a] The substrains were grown in an identical manner with a limited number of passages, as described in the Materials and Methods section. All substrains had one copy of IS*6110*. RDs are based on the work of Mostowy et al. (22).

against which novel vaccine candidates are compared (18), and the mouse model has also been used to examine differences between BCG substrains. By using genetically homogeneous populations of inbred mouse strains, differences in the immune response to BCG substrains can be investigated. Recent investigations of multiple BCG substrains have demonstrated that the mouse model is able to discriminate between substrains on the basis of their ability to reduce the bacterial burden and on the basis of postchallenge cytokine studies (9).

In the current study, three BCG substrains were used to determine if the magnitude of the adaptive immune response and if their ability to reduce the *M. tuberculosis* burden in C57BL/6 mice varied with the RDs within substrains. This strain of mouse was chosen because it is commonly used to test novel vaccine candidates, and information from this study will contribute to the interpretation of data for candidate vaccines (18, 26). The three substrains tested were BCG Pasteur, Sweden, and Connaught, which, according to the lineage described by Behr and colleagues (1), span a wide range of BCG strains from the early (pre-RD2) and late (RD2) groups (Table 1). The substrains were chosen on the basis of their genetic diversity and, thus, were intended to be representative of the diverse set of substrains with deletions that represent the currently known deletions.

C57BL/6 mice were vaccinated with each BCG substrain; and lung and spleen cells were analyzed at 14, 21, and 42 days postvaccination for the number of activated CD4 and CD8 T cells (including antigen-specific CD8 T cells). Separate groups of vaccinated mice were also challenged with a virulent *M. tuberculosis* strain at each of these time points and were killed 30 days later to determine if the reduction in the bacterial burden in the target organs varied over time and if this correlated with the level of immunity at those times.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Charles River (Wilmington, MA) and were maintained at the Colorado State University (CSU) Laboratory Animal Resources Facility in individual positive-ventilation cages (Thoren Industries, Hazleton, PA). The mice were given sterile food and water ad libitum. The mice were free of common viral pathogens, as determined by routine screening of sentinel mice performed at the Research Animal Diagnostic Laboratory, University of Missouri, Columbia.

Mycobacteria. Before they were sent to CSU, *M. bovis* BCG Pasteur, Sweden, and Connaught were first grown on potato soaked in Sauton's medium and were subsequently grown as a surface pellicle on Sauton's liquid medium at 37.5°C (16). Concentrated stocks were stored in Sauton's liquid medium at −20°C or glycerol at −80°C. At CSU, the substrains were then passaged twice to expand the cultures as working stocks in Proskauer and Beck (P&B) medium containing 0.05% Tween 80 to mid-log phase, and stocks of 1-ml aliquots were frozen at −80°C until they were used. After several days at −80°C, three vials of each substrain were thawed and plated for determination of the numbers of viable bacteria. At the same time, the total number of organisms in each vial was determined with a Petroff-Hauser counting chamber, and this number was then compared to the number of CFU. All BCG substrains had >90% viable organisms. Each strain was genetically characterized and its identity was confirmed, according to the protocols previously established by Bedwell et al. (1). *M. tuberculosis* H37Rv (Trudeau Mycobacterial Culture Collection strain 102) was grown as a pellicle on P&B medium and then passaged three times in P&B medium containing 0.05% Tween 80 to mid-log phase, and stocks were aliquoted and frozen at −80°C until they were used.

Vaccination and low-dose aerosol infection. The BCG substrains were diluted in pyrogen-free sterile saline, and the mice were vaccinated once with 10^6 CFU of the BCG substrains by the subcutaneous route in the scruff of the neck. The concentration of each inoculum was checked by plating 10-fold serial dilutions on Middlebrook 7H11 agar (Difco Laboratories, Detroit, MI) and incubating the plates at 37°C for 14 to 21 days. To determine the dissemination of BCG, five mice per group were killed at 14, 21, and 42 days postvaccination; and the lung, spleen, and draining lymph nodes (cervical, mediastinal, and inguinal) were excised, pooled, homogenized in saline, and plated in total on Middlebrook 7H11 agar. In addition, at 14, 21, and 42 days postvaccination, five mice per group were challenged with a low-dose aerosol of virulent *M. tuberculosis* H37Rv by use of the Middlebrook inhalation exposure system (Glas-Col, Terre Haute, IN). To assess the effect of vaccination on the growth of *M. tuberculosis* at each time point, the mice were killed at 30 days postchallenge; and the lungs and spleen were excised, homogenized in sterile saline, and then plated on Middlebrook 7H11 agar in 10-fold serial dilutions.

Flow cytometric analysis of single-cell suspensions. At the prescribed times postvaccination as well as postchallenge, the mice were killed and the lungs and spleen were excised. Single-cell suspensions were prepared as described previously (17) and analyzed for their cell surface phenotype by using monoclonal antibodies to CD4 (clone GK1.5), CD8α (clone 53-6.7), CD44 (clone IM7), CD45RB (clone 16A), and CD62L (clone MEL-14). A phycoerythrin Mtb32 *H-2D*^{b} major histocompatibility complex class I tetramer (Beckman Coulter Immunomics, San Diego, CA) specific for the epitope GAPINSATAM was used to identify Mtb32-specific CD8 T cells (17). The frequency of IFN-γ-producing cells in each subpopulation was determined by intracellular cytokine staining with an anti-IFN-γ antibody (clone XMG1.2). The cell populations were analyzed with a FACSCalibur dual-laser flow cytometer (BD Biosciences, Mountain View, CA), and the data were analyzed with CellQuest software (BD Biosciences). All antibodies were purchased from BD Pharmingen.

Enzyme-linked immunospot (ELISPOT) assay for IFN-γ-producing cells. Cells were prepared from the spleens of vaccinated mice at 42 days postvaccination. The cells were cultured in complete RPMI 1640 (10% fetal bovine serum, penicillin-streptomycin, and L-glutamine) in 96-well MultiScreen HTS IP sterile plates (Millipore, Bedford, MA) precoated with anti-IFN-γ antibody, according to the manufacturer's protocol (eBioscience, San Diego, CA). To determine if there were differences in the numbers of IFN-γ-producing T cells in spleen cells from mice vaccinated with the different BCG substrains, spleen cells isolated from each group of vaccinated mice were stimulated with *M. tuberculosis* H37Rv for 48 h. The plates were developed with horseradish peroxidase-conjugated anti-IFN-γ-detecting antibody, followed by streptavidin-horseradish peroxidase and 3-amino-9-ethylcarbazole substrate (Sigma, St. Louis, MO), and the spots were counted with an Immunospot reader (Cellular Technology Limited, Cleveland, OH).

Stimulation of BMDMs. Bone marrow-derived macrophages (BMDMs) from the mice were infected with the BCG substrains at three multiplicities of infection (MOIs; 1:1, 3:1, and 10:1) in triplicate cultures, as described previously (11). The BMDMs were incubated with the bacteria at 37°C in 5% CO_2 for 4 h, washed twice with phosphate-buffered saline (PBS), and incubated in infection medium with gentamicin for an additional 14 h at 37°C in 5% CO_2. The culture supernatants were collected at the end of infection and were assayed for the levels of the interleukin-12 (IL-12) p40 subunit by enzyme-linked immunosorbent assay (ELISA; BD Biosciences). For each BCG substrain, the ELISA readings from three different wells were averaged. The experiments were performed on four separate occasions.

Statistical analyses. The data were initially analyzed for normality and were then subjected to multiple-group analysis by the use of one-way analysis of variance with the Bonferroni *t* test by using SigmaStat software (San Jose, CA) to test the differences. Analysis of the numbers of CFU (except for the BCG dissemination data, for which the raw numbers were used) and the cell numbers was performed with $\log_{10}$-transformed data.

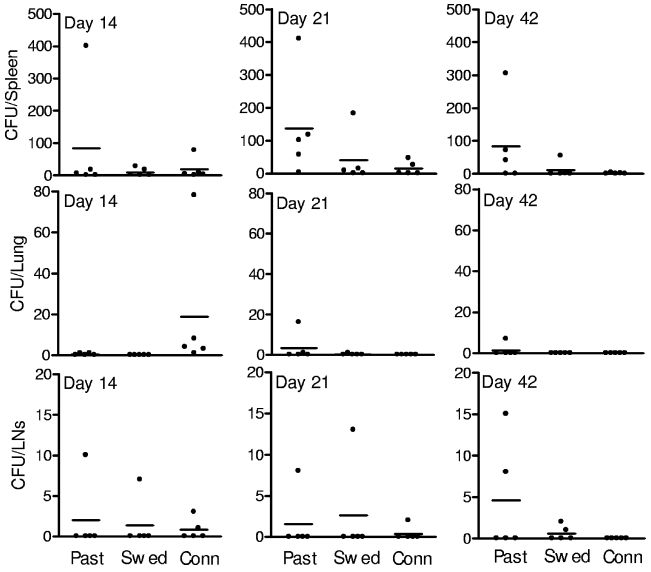

FIG. 1. Dissemination of the BCG substrains to the lung, spleen, and lymph nodes after subcutaneous inoculation. Mice ($n = 5$ mice per group per time point) were inoculated subcutaneously with 10^6 CFU of each substrain; and the numbers of CFU in the lung, spleen, and lymph nodes (pooled cervical, mediastinal, and inguinal) were assessed at 14, 21, and 42 days postinoculation. The organs were homogenized in sterile saline and then plated on Middlebrook 7H11 agar. The results are for five mice per group per time point and are representative of those from two experiments. Past, Swed, and Conn, substrains Pasteur, Sweden, and Connaught, respectively.

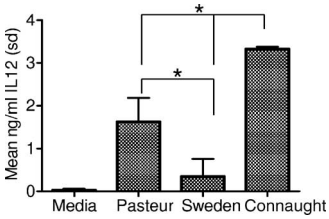

FIG. 2. Mean and standard deviation (sd) of the IL-12 p40 concentrations in supernatants from BMDMs infected with BCG Pasteur, Connaught, or Sweden at an MOI of 1:1. BMDMs from C57BL/6 mice were infected with each substrain, the supernatants were taken after 24 h, and the level of IL-12 p40 was determined by ELISA. *, $P < 0.05$. The experiments were performed on four separate occasions.

RESULTS

Dissemination of BCG after subcutaneous vaccination. To determine the kinetics of dissemination from the site of vaccination, mice were inoculated in the scruff of the neck with 10^6 CFU of each BCG substrain and the numbers of CFU in the lymph nodes (cervical, mediastinal, and inguinal), lung, and spleen were determined at 14, 21, and 42 days postvaccination. In general, there were significantly greater numbers of BCG organisms in the spleen than in either the lungs or the lymph nodes ($P < 0.001$), but the numbers of organisms in the spleen varied over time, without a consistent pattern (Fig. 1). For BCG Pasteur, the number of splenic CFU increased at day 21 but was not significantly different from the numbers observed at days 14 and 42. The number of CFU observed with BCG Sweden was generally greater at 21 days postvaccination and was undetectable at 42 days in all but one mouse (Fig. 1). The level of dissemination of BCG Connaught to the spleen was similar to that observed for BCG Sweden. Very few numbers of CFU of BCG Pasteur or Sweden were detected in the lungs, while none could be detected in the BCG Connaught-inoculated mice beyond day 14. Similarly, small numbers of BCG CFU were detected in the sampled lymph nodes, suggesting that lymphatic dissemination had occurred for all substrains. Overall, the data suggest that BCG failed to grow significantly after subcutaneous vaccination, as fewer organisms than were present in the inoculum were detected.

Induction of IL-12 by BCG substrains. Given that the BCG substrains disseminated, we next wanted to determine if they differed in their ability to induce IL-12, an important cytokine required for the induction of the Th1 immune response, using BMDMs that were isolated and tested in vitro and that were infected at multiple MOIs. Figure 2 represents the data for an MOI of 1:1 and shows that BCG Connaught induced significantly higher levels of IL-12 than BCG Pasteur ($P < 0.05$) and that BCG Pasteur induced significantly more IL-12 than BCG Sweden ($P < 0.05$). Similar results were obtained for cultures in which the MOIs were 3:1 and 10:1, as well as in repeat experiments with these inocula (data not shown).

Pulmonary immune responses generated by BCG substrains. Following the subcutaneous vaccination of mice with the BCG substrains, there was a moderate but significantly increased number of pulmonary $CD4^+$ T cells (Fig. 3A; $P < 0.05$) and $CD8^+$ T cells (Fig. 3B; $P < 0.05$) above the number for the sham (PBS)-treated mice as early as day 14. After this early increase, the numbers of $CD4^+$ and $CD8^+$ T cells in the lungs of vaccinated mice did not differ significantly from those in the lungs of the PBS-treated mice (Fig. 3A and B) for the duration of the experiment.

To determine if the early increase in pulmonary $CD4^+$ and $CD8^+$ T-cell numbers correlated with increased effector cell function, the lung cells were also assessed for IFN-γ production by intracellular cytokine staining (Fig. 4). The number of IFN-γ-producing $CD4^+$ T cells in the lungs of BCG-vaccinated mice was significantly greater than the number in PBS-treated mice at all time points tested (Fig. 4A; $P < 0.001$), but there was no difference between the BCG substrains. While the number of $CD8^+$ IFN-γ-producing T cells in all inoculated groups was significantly elevated above the number in the PBS-treated mice at day 14 (Fig. 4B; $P < 0.05$), the increase elicited with BCG Connaught was significantly larger than what was seen with either BCG Pasteur or Sweden at this time point (Fig. 4B; $P < 0.05$). At day 21, while vaccination with BCG Pasteur and Sweden maintained elevated numbers of $CD8^+$ IFN-γ-producing T cells ($P < 0.05$), the numbers of these cells again declined in mice vaccinated with BCG Connaught. No significant differences were observed among the groups at day 42.

Secondary immune response varies with BCG substrain. Given that BCG vaccination has been shown to provide limited and variable protection against adult pulmonary tuberculosis and that the genetic compositions of the different BCG vaccines strains used clinically may contribute to these limitations, we next wanted to determine if the magnitude of the immune response generated by each substrain differed in the presence

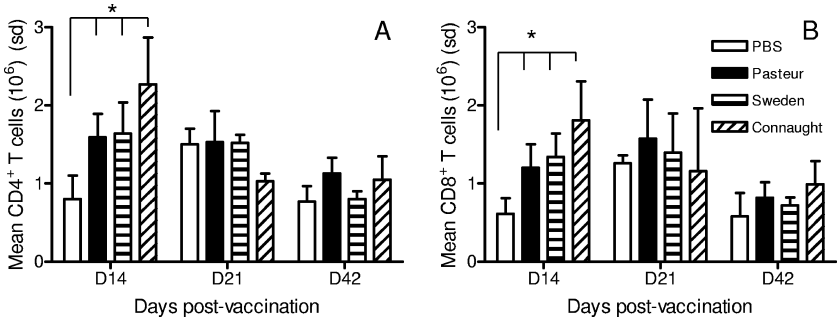

FIG. 3. Mean and standard deviation (sd) of the total number of $CD4^+$ T cells (A) and $CD8^+$ T cells (B) in the lungs of vaccinated mice at days 14 (D14), 21 (D21), and 42 (D42) postvaccination. Lung single-cell suspensions ($n = 5$ mice per group) were subjected to flow cytometric analysis after incubation of the cells with anti-CD3/anti-CD4 and anti-CD3/anti-CD8 antibodies. *, $P < 0.05$. Data are representative of those from two experiments.

of *M. tuberculosis*. Spleen cells from mice which had been vaccinated with the different BCG substrains 42 days previously were stimulated in vitro with viable *M. tuberculosis* H37Rv cells or the BCG substrains with which they were vaccinated, and the number of IFN-γ-producing T cells was assessed by the ELISPOT assay (Fig. 5). In response to H37Rv (Fig. 5A), spleen cells from BCG Pasteur- and BCG Connaught-vaccinated mice produced similar numbers of IFN-γ-producing cells, and these numbers were significantly greater than the numbers measured in cells from mice vaccinated with BCG Sweden ($P < 0.05$). The number of IFN-γ-producing T cells, after in vitro stimulation with the same BCG substrain used for the vaccination (Fig. 5B), was elevated in the BCG Connaught- and BCG Pasteur-inoculated mice, although only the number in the mice inoculated with BCG Connaught was significantly increased above that in mice inoculated with BCG Sweden ($P < 0.05$).

Vaccination-induced BCG antigen-specific $CD8^+$ T cells. Our data have shown that the total number of pulmonary $CD8^+$ T cells was elevated above that in the PBS-treated mice within 14 days of vaccination for all the BCG substrains. To examine whether this increase corresponded to an increase in antigen-specific $CD8^+$ T cells, we used a tetramer reagent specific for a defined epitope in the Mtb32 antigen (17). $CD8^+$ lung cells from vaccinated mice were analyzed by flow cytometry for the expression of the T-cell receptor to bind to the tetramer at days 14, 21, and 42 (Fig. 6). Each substrain differed in its ability to induce lung $CD8^+$ Mtb32-specific T cells, with BCG Sweden unable to induce significantly greater numbers of cells than PBS treatment and with BCG Pasteur inducing significantly elevated numbers only at day 21 ($P < 0.05$ versus the results for PBS-treated and BCG Sweden-inoculated mcie). BCG Connaught induced the greatest increase in Mtb32-specific cells at all time points ($P < 0.05$ versus the results for PBS-treated mice), although at day 42 the number of cells was reduced significantly.

Reduction of bacterial burden and immune response after low-dose aerosol challenge with virulent *M. tuberculosis*. Mice vaccinated with each BCG substrain were exposed on days 14, 21, and 42 days to a low-dose aerosol challenge with virulent strain *M. tuberculosis* H37Rv. Thirty days later, the lungs and spleens were harvested and the numbers of CFU were determined. Regardless of the substrain used for vaccination or the time to challenge, all BCG substrain-vaccinated mice had significantly fewer numbers of CFU in their lungs and spleens (data not shown) at day 30 postinfection than the PBS-treated mice (Fig. 7; $P < 0.001$).

Effector cell numbers in the lungs of infected mice, as determined by measurement of the $CD44^{hi}$ $CD62L^{lo}$ markers on CD4 and CD8 T cells, were significantly elevated ($P < 0.05$) in mice receiving PBS compared to the numbers in mice vaccinated with the BCG substrains (Fig. 8). There was no difference in the effector-T-cell response between the BCG substrain-vaccinated groups for either CD4 or CD8 T cells. To

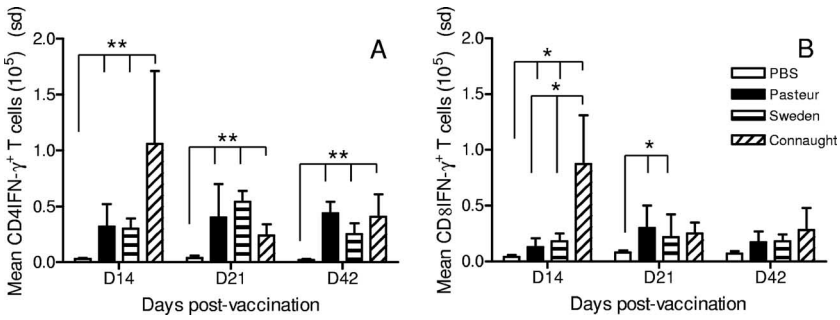

FIG. 4. Mean number and standard deviation (sd) of $CD4^+$ (A) and $CD8^+$ (B) IFN-γ-producing T cells in the lungs of vaccinated mice at days 14 (D14), 21 (D21), and 42 (D42) postvaccination, as determined by intracellular cytokine staining. Lung single-cell suspensions ($n = 5$ mice per group) were subjected to flow cytometric analysis after in vitro restimulation with anti-CD3/anti-CD28 antibodies. *, $P < 0.05$; **, $P < 0.001$. Data are representative of those from two experiments.

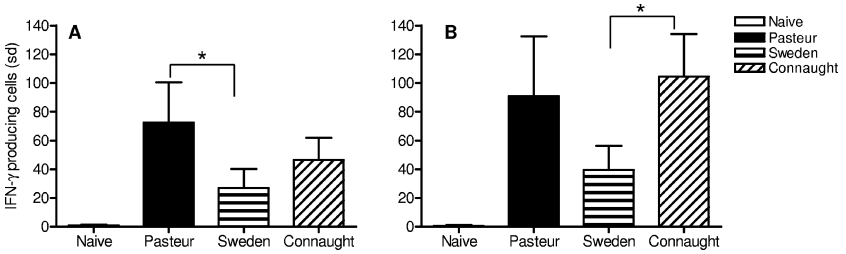

FIG. 5. Mean number and standard deviation (sd) of splenic IFN-γ-producing T cells per 5×10^5 T cells after in vitro stimulation. Splenocytes obtained from vaccinated mice at day 42 postvaccination were stimulated in vitro with *M. tuberculosis* H37Rv (A) or with the same BCG substrain with which they were vaccinated (B) for 24 h, and the number of BCG-specific T cells producing IFN-γ was determined by ELISPOT assay. The results are for three mice per group per time point and are representative of those from two experiments. *, $P < 0.05$.

determine if the reduced immune response in BCG-vaccinated mice was also antigen specific, we examined the Mtb32-specific $CD8^+$ T cells in the lungs of infected mice (Fig. 8). Greater numbers of antigen-specific $CD8^+$ T cells were observed in the lungs of PBS-treated, challenged mice, but this increase was not statistically significant except at day 21 postvaccination for mice vaccinated with BCG Pasteur and Sweden ($P < 0.05$). This may reflect the relatively higher mycobacterial burden in the lungs of PBS-treated mice at the time of sampling.

DISCUSSION

In the current study we used the C57BL/6 mouse model to determine whether three BCG substrains can be distinguished on the basis of the immune response that they elicit in mice after vaccination and challenge. The immune response generated by each substrain of BCG did not correlate with the extent of the genomic deletions. In fact, as determined by the use of various quantitative measures of immunity after vaccination, BCG Sweden, which had the greatest genomic content, was the least capable of inducing activated T cells during the early phase after vaccination. All strains were similarly effective in reducing the mycobacterial burden in the lungs and spleens after low-dose aerosol infection with a virulent *M. tuberculosis* strain. Taken together the data suggest that (i) the C57BL/6 mouse model may be relatively insensitive for discriminating between the capacities of different BCG substrains to reduce the *M. tuberculosis* infection, (ii) the immune response induced by BCG was not related to the extent of genomic deletion but each BCG substrain induced a response that was sufficient to reduce the bacterial burden, and (iii) a much more profound deletion of the genome would be required before the mouse model could detect differences in the capacities of the BCG substrains to reduce the mycobacterial burden.

The ability of BCG to spread to primary lymphoid organs is a major factor in determining whether it can induce an adaptive immune response (28). The fact that BCG was detected in lymphoid organs at various times after inoculation, even at relatively low numbers, was sufficient to allow the induction of an adaptive immune response. Our observations suggest that after subcutaneous inoculation, only a fraction of the inoculated BCG could be detected in the lymph nodes sampled, with BCG also being found in the spleens and lungs at all time points examined (Fig. 1), indicating that either the BCG had spread to other organs (such as the liver, which was not examined) or the majority of the inoculating dose was eliminated prior to day 14. A previous report showed that the majority of the BCG Danish 1331 inoculum was detected in draining lymph nodes and that this persisted for up to 5 months (25). Since the ability to spread to other organs was observed to various degrees with all the BCG substrains (Fig. 1), this would suggest that the RD2, nRD18, RD14, and RD8 regions are not essential for dissemination.

The total number of pulmonary $CD4^+$ and $CD8^+$ T cells in vaccinated mice (Fig. 3, 4, and 5), which expressed an activated phenotype, increased within 2 weeks but then receded to background levels from day 21 onwards. Given that none of the

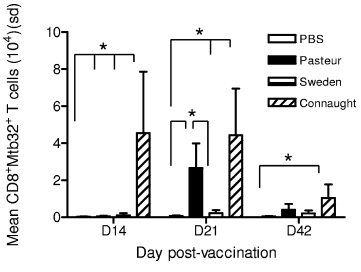

FIG. 6. Mean number and standard deviation (sd) of Mtb32-specific CD8 T cells in the lungs of BCG vaccinated mice were determined at days 14 (D14), 21 (D21), and 42 (D42) postvaccination. Cells were stained with a peptide-specific major histocompatibility complex class I tetramer in conjunction with an anti-CD8 monoclonal antibody. The results are for five mice per group and are representative of those from two experiments. *, $P < 0.05$.

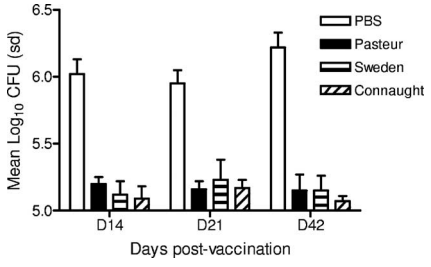

FIG. 7. Mean number and standard deviation (sd) of CFU in the lungs of BCG vaccinated mice at day 30 after low-dose aerosol challenge with virulent strain *M. tuberculosis* H37Rv. The mice were challenged at days 14 (D14), 21 (D21), and 42 (D42) postvaccination. The numbers of CFU were determined by plating serial 10-fold dilutions of organ homogenates on Middlebrook 7H11 agar and incubation for 14 to 21 days at 37°C. The results are for five mice per group per time point and are representative of those from two separate experiments.

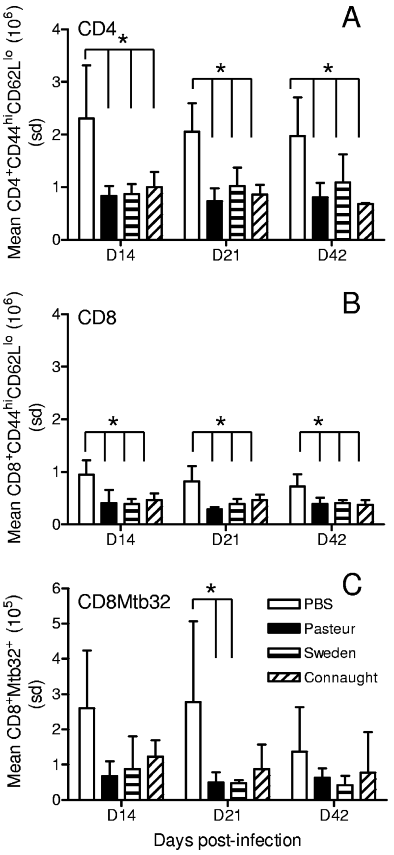

FIG. 8. Mean and standard deviation (sd) of the total numbers of pulmonary $CD4^+$ effector T cells ($CD44^{hi}$ $CD62L^{lo}$) (A), $CD8^+$ effector T cells ($CD44^{hi}$ $CD62L^{lo}$) (B), and antigen-specific $CD8^+$ Mtb32 antigen-positive T cells (C) at day 30 after aerosol challenge with *M. tuberculosis* H37Rv. The mice were challenged at days 14 (D14), 21 (D21), and 42 (D42) postvaccination. The results are for five mice per group per time point and are representative of those from two experiments. *, $P < 0.05$.

BCG substrains appeared to multiply significantly, particularly within the lungs, it was not surprising that the number of effector cells declined following the initial increase. Thus, effector T cells circulated through the lung after vaccination and in the absence of any further stimuli either migrated out of the lung or underwent apoptosis. Furthermore, these results suggest that strategies involving BCG priming and boosting with recombinant antigen would be most effective at time points greater than 21 days postimmunization, since the effector-T-cell response had significantly declined by this time. Indeed, others have shown that a boost given 14 weeks or later after BCG administration can increase the reduction in the mycobacterial burden compared to that achieved after BCG administration alone (14).

Analysis of the spleen-derived IFN-γ-producing T-cell frequencies from BCG-vaccinated mice when they were stimulated in vitro with *M. tuberculosis* (Fig. 5A) showed that mice vaccinated with each of the substrains induced greater numbers than naïve mice, with the highest response being in BCG Pasteur-vaccinated mice at day 42 postvaccination and with the lowest response being observed in BCG Sweden-vaccinated mice. These data, in conjunction with the CFU data (Fig. 7), suggest that a frequency of approximately 20 IFN-γ-producing T cells per 5×10^5 T cells, as observed with BCG Sweden, which induced significantly fewer T cells than BCG Pasteur did, was sufficient to reduce the bacterial burden by approximately 0.75 to 1 $\log_{10}$ CFU. Despite these statistically significant differences in the IFN-γ responses, there was no correlation with a reduction of the mycobacterial burden, as there was no difference in the abilities of the different BCG substrains to reduce the mycobacterial burden (Fig. 7). Interestingly, although *M. tuberculosis* has a greater repertoire of antigens than BCG, there were fewer IFN-γ-producing T cells when spleen cells from vaccinated mice were stimulated in vitro with *M. tuberculosis* (Fig. 5B).

The current study is not the first to examine BCG substrains. In early studies performed with guinea pigs, Smith et al. (31) revealed differences in the abilities of 10 BCG substrains to reduce the number of CFU after low-dose aerosol infection with virulent mycobacteria. However, since the identity of the strains was not listed, it was not possible to identify which strains were the most potent. A study by Lagranderie et al. (20) with five different BCG substrains supported the fact that there were differences in the immunogenicities of various BCG vaccine strains and that these differences may play a major role in BCG vaccination efficiency. Castillo-Rodal et al. (9), using 10 geographically distinct substrains of BCG in a high-dose intratracheal infection model with BALB/c mice, showed that postchallenge there were differences in the abilities of the strains to induce specific cytokines. These data are in contrast to our observations, since we did not find IL-10-secreting cells postchallenge in the lungs of vaccinated mice (data not shown), while the previous study found significantly elevated levels of IL-10 in the lungs of mice vaccinated with BCG Sweden and then challenged. This difference may be explained by the fact that the mouse strain used was different from the one used here. Unfortunately, in the study of Castillo-Rodal (9), there was no analysis of the immune response induced by the BCG strains, other than the delayed-type hypersensitivity response (9). A meta-analysis of the BCG strains used in efficacy trials with humans suggested that the BCG strain used for vaccination is not a significant determinant of the overall efficacy for the prevention of tuberculosis (8).

Fundamentally, the data from the C57BL/6 mouse model suggest that extrinsic factors other than immune response induction may be affecting the outcome of BCG vaccination in humans. The mouse model clearly tells us that the number of CFU obtained after infection and the immune response generated by BCG are not related and that we need to better understand the immune response in humans as well as in the mouse model. Hence, the question remains as to whether the mouse model, irrespective of the strain of mouse used, is the best model to be used to answer questions raised from the clinical experience.

The inability of the C57BL/6 mouse strain to discriminate between the BCG substrains used in this study on the basis of the number of CFU may be due to several factors. One factor may be that the different immunogenic antigens encoded in the RD region may be important immunologically but not sufficient to contribute to a greater extent in the reduction in the number of CFU. Alternatively, a sufficient number of other antigens that can be classified as immunodominant may be

present in the remaining genome and, thus, produce an immune response regardless of the extent of genome deletion. Certainly, our data would suggest that in the mouse model, the presence of fewer deleted regions does not provide an immunologic advantage over the presence of larger amounts of intact genomic DNA. Others have shown that monitoring of the number of CFU at later time points (4 months postchallenge) may be a better discriminator to show differences in the number of CFU; thus, determining the number of CFU at day 30 postchallenge or later may have uncovered differences between substrains (29).

Assessment of the number of CFU in the C57BL/6 mouse model could not significantly discriminate between these three BCG substrains, and each provided an approximately 1-$\log_{10}$ reduction in the mycobacterial burden, regardless of the time after vaccination at which the burden was tested. Although T cells are known to be essential during infection with *M. tuberculosis*, our results suggest that the possession of greater numbers of these cells beyond a threshold amount confers no additional advantage in reducing the bacterial load after infection. A sufficient number of T cells was generated throughout the time course to limit the growth of *M. tuberculosis* in the lungs, and again, any further increase in the number of T cells may not provide an increased ability to reduce the bacterial burden. It may also be possible that the inoculating dose used in this study predisposed the mice to reducing the bacterial burden by 1 $\log_{10}$, regardless of the amount of genomic material present. Previous studies have shown that the ability of a BCG substrain to significantly reduce the bacterial burden was not dependent on the dose (15), while others have reported that the dose can affect the type (a Th1 type versus a Th2 type) of immune response generated (27).

Overall, this study has shown that although BCG substrains with various degrees of genomic deletions could be differentiated on the basis of the magnitude of the immune response that they elicited after vaccination in mice, the data presented do not support the hypothesis that BCG substrains encoding fewer antigens were less able to reduce the mycobacterial burden in a mouse model of infection. In fact, in some assays the strain of BCG containing the most genomic deletions (BCG Connaught) induced the greatest response.

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